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A protective role for FGF-23 in local defence against disrupted arterial wall integrity?

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Abstract

Increasing interest is focusing on the role of the FGF-23/Klotho axis in mediating vascular calcification. However, the underpinning mechanisms have yet to be fully elucidated. Murine VSMCs were cultured in calcifying medium for a 21d period. FGF-23 mRNA expression was significantly up-regulated by 7d (1.63 fold; P<0.001), with a concomitant increase in protein expression. mRNA and protein expression of both FGFR1 and Klotho were confirmed. Increased FGF-23 and Klotho protein expression was also observed in the calcified media of *Enpp1*$^{-/-}$ mouse aortic tissue. Reduced calcium deposition was observed in calcifying VSMCs cultured with recombinant FGF-23 (10ng/ml; 28.1% decrease; P<0.01). Calcifying VSMCs treated with PD173074, an inhibitor of FGFR1 and FGFR3, showed significantly increased calcification (50nM; 87.8% increase; P<0.001). FGF-23 exposure induced phosphorylation of ERK1/2. Treatment with FGF-23 in combination with PD98059, an ERK1/2 inhibitor, significantly increased VSMC calcification (10μM; 41.3% increase; P<0.01). Use of FGF-23 may represent a novel therapeutic strategy for inhibiting vascular calcification.

Keywords

vascular calcification; vascular smooth muscle cells; *Enpp1*; FGF-23

1. Introduction

Vascular calcification is a marker of increased cardiovascular risk in ageing, and in a number of diseases including diabetes, atherosclerosis and chronic kidney disease (CKD) (Demer and Tintut, 2008; Mackenzie and MacRae, 2011; Zhu et al.,2012). Although condition-specific factors are likely to drive the calcification process, the etiology of mineral accumulation within the vasculature shares many similarities with that of bone formation (Demer and Tintut, 2008; Shroff and Shanahan, 2007). Indeed, a number of studies have reported that vascular smooth muscle cells (VSMCs), the predominant cell type involved in vascular calcification, can undergo phenotypic transition to osteoblastic, chondrocytic and osteocytic cells in a calcified environment (Speer et al., 2005; Zhu et al., 2011). Furthermore, it has been demonstrated that phosphate accelerates this phenotypic trans-differentiation, evident in the loss of characteristic smooth muscle markers and the

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development of osteoblastic features, such as the expression of tissue-nonspecific alkaline phosphatase, P_iT-1, osteocalcin and osteopontin, and osteocyte markers including sclerostin and E11 (Speer et al., 2009; Zhu et al., 2011). Vascular calcification also involves the reciprocal loss of recognised calcification suppressors, such as inorganic pyrophosphate (PP_i), MGP and fetuin A (Murshed et al., 2005; Rutsch et al., 2003).

The family of Fibroblast Growth Factors (FGFs) consists of 23 proteins that regulate cell proliferation, migration, differentiation and survival (Eswarakumar et al., 2005). FGF-23, the most recently discovered FGF, is produced by osteocytes in bone and regulates phosphate homeostasis via signalling through its receptors (mainly FGFR1) in the presence of Klotho, its cofactor in the kidney and parathyroid glands (Kurosu et al., 2006; Shimada et al., 2001; Urakawa et al., 2006). The primary physiological actions of FGF-23 are to augment phosphaturia by downregulating the expression of type IIa and IIc sodium-phosphate transporters within the renal proximal tubular cells and to decrease circulating concentrations of 1,25-dihydroxyvitamin D_3 via inhibition of 1α hydroxylase activity (Kurosu et al., 2006; Wolf, 2010). FGF-23 also negatively regulates parathyroid hormone (PTH) secretion (Ben-Dov et al., 2007).

Increasing interest is focusing on the role of the FGF-23/Klotho axis in mediating vascular calcification. A direct correlation between FGF-23 circulating levels and the extent of aortic calcium deposition in mice fed a high-phosphate diet has been recently demostrated (El Abbadi et. al., 2009). An association between FGF-23 levels and calcium accumulation in the arteries of dialysis patients has also been reported (Srivaths et al., 2011). Increased circulating FGF-23 levels have also been observed in the *Enpp1*$^{-/-}$ mouse model of medial vascular calcification (Mackenzie et al., 2012a), as well as in patients with hypophosphatemic rickets resulting from a loss of function mutation in the *ENPP1* gene (Lorenz-Depiereux et al., 2010). In patients with CKD, increased FGF-23 plasma levels have been linked to a decrease in kidney function, the presence of vascular damage and an increased risk of cardiovascular mortality (Isakova et al., 2011; Nashrallah et al., 2010; Shrivaths et al., 2011; Yilmaz et al., 2010). However, both clinical and basic studies have demonstrated conflicting evidence as to whether FGF-23 imparts a protective or a harmful role on the vasculature during stress. FGF-23 may therefore maintain vascular health at physiological levels, and may only at high circulating concentrations exert harmful effects. Interestingly, recent studies have suggested that FGF-23 directly inhibits vascular calcification (Lim et al., 2012; Razzaque and Lanske, 2007, Shalhoub et al., 2012). However it has also been suggested that elevated FGF-23 concentrations may stimulate vascular calcification by acting directly on the vascular wall to induce a local reduction of Klotho (Donate-Correa et al., 2011). Therefore, in the present study, we have undertaken *in vitro* and *ex vivo* murine VSMC calcification studies to provide fundamental insights into the expression profiles of FGF-23 during vascular calcification. Further investigations have provided novel insights into the functional role and underpinning mechanisms of FGF-23 in protecting VSMCs from pathological calcification.

2. Materials and Methods

2.1. Enpp1$^{-/-}$ mice

Enpp1$^{-/-}$ mice were generated and characterised as previously described (Sali et al., 1999). Genotyping was performed by a commercial genotyping service (Genetyper, New York, USA) using genomic DNA isolated from ear clips. All animal experiments were approved by The Roslin Institute's Animal Users Committee and the animals were maintained in accordance with Home Office guidelines for the care and use of laboratory animals.

2.2. Primary murine VSMC isolation

Primary VSMCs were isolated from 5-week old wild-type (WT) C57BL/6 mice as previously described (Johnson et al., 2005). Briefly, after removal of adventitia, the aorta was cut open to expose the endothelial layer. Tissues from eight animals were pooled for digestion with 1mg/ml trypsin for 10min in order to remove any remaining adventitia and endothelium. Following a further overnight incubation at 37°C in a humidified atmosphere of 95% air/5% CO_2 in growth medium consisting of α-MEM (Invitrogen, Paisley, UK) supplemented with 10% FBS (Invitrogen) and 1% gentamicin (Invitrogen), tissues were then digested with 425U/ml collagenase type II (Worthington Biochemical Corporation, Lakewood, USA) for 5 h. Isolated VSMCs were expanded in growth medium for two passages in T25 tissue culture flasks (Greiner Bio-one, GmbH, Frickenhausen, Baden-Wurttemberg, Germany) coated with 0.25μg/cm^2 murine laminin (Sigma, Poole, UK) to promote maintenance of the contractile differentiation state (Johnson et al., 2008).

2.3. Cell culture

Primary VSMCs were seeded in growth medium at a density of 1.5×10^4/cm^2 in multi-well plates. At confluency (day 0), VSMCs were cultured in growth medium supplemented with 2.5 mM β-glycerophosphate (βGP) (Sigma) and 50 μg/ml ascorbic acid (AA) (Sigma) or 3mM Na_2HPO_4/NaH_2PO_4 (P_i) (Sigma) for up to 21 d to induce calcification. Cells were maintained in 95% air/5% CO_2 and the medium was changed every third/fourth day.

Recombinant mouse FGF-23 (R&D Systems, Abingdon, UK) at 10–50 ng/ml was added to cultures at confluence for up to 9 days. PD98059 (Sigma) at 10 μM and PD173074 (Source Bioscience, Nottingham, UK) at 10 and 50nM were also added at confluence in 0.1% DMSO to inhibit Erk1/2 signalling and FGFR1, respectively. Control cultures received 0.1% DMSO only. Cell viability was assessed using a commercially available kit (Alamar Blue; Invitrogen).

2.4. Detection of calcification

Calcium deposition was evaluated by staining the cell-matrix monolayer with alizarin red (Sigma) as previous described (MacRae et al., 2010). In brief, VSMCs were washed twice with phosphate buffered saline (PBS), fixed in ice-cooled 4% paraformaldehyde (PFA) for 5 min at 4°C, stained with 2% alizarin red (pH 4.2) for 10 min at room temperature and rinsed with distilled water. Alizarin red stained cultures were extracted with 10% cetylpyridium chloride for 10 min and the O.D. was determined at 570 nm by spectrophotometery (Multiskan Ascent, Thermo Electron Corporation, Vantaa, Finland). Calcium deposition in VSMCs was also assessed by HCL leaching. Cells were decalcified in 0.6N HCL overnight and free calcium determined colorimetrically by a stable interaction with phenolsulphonethalein using a commercially available kit (Randox Laboratories Ltd., County Antrim, UK) and corrected for total protein concentration, following extraction using 1mM NaOH in 0.1%SDS (Bio-Rad Laboratories Ltd, Hemel Hempstead, UK).

2.5. Alkaline phosphatase (ALP) activity

Cell layers were lysed with 0·9% NaCl and 0·2% Triton X-100 and centrifuged at 12 000 g for 15 min at 4°C. The supernatant was assayed for protein content and ALP activity. Enzyme activity was determined by measuring the cleavage of 10 mM p-nitrophenyl phosphate (pNPP) at 410 nm using a commercially available kit (Thermo Trace, Melbourne, Australia). Total ALP activity was expressed as nmoles pNPP hydrolysed/min/mg protein (Mackenzie et al., 2011; Zhu et al., 2011).

2.6. Real-Time-Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from VSMCs using RNeasy total RNA (Qiagen Ltd, Crawley, West Sussex, UK), according to manufacturer's instructions. RNA was quantified and reversed transcribed as previously described (MacRae et al., 2006a; MacRae, et al., 2009). All genes were analyzed with the SYBR green detection method (Roche, East Sussex, UK) using the Stratagene Mx3000P real-time QPCR system (Stratagene, CA, USA). Each PCR was run in triplicate. All gene expression data were normalized against *Gapdh* and the control values expressed as 1 to indicate a precise fold change value for each gene of interest. Primers for *Runx2* forward 5′-ACC ATA ACA GTC TTC ACA AAT CCT-3 and reverse 5′CAG GCG ATC AGA GAA CAA ACT A-3, *Pit-1* forward 5′-CAC TCA TGT CCA TCT CAG ACT-3 and reverse 5′-CGT GCC AAA GAA GGT GAA C-3, *Fgf-23* forward 5′-GGA TCT CCA CGG CAA CAT TT-3 and reverse 5′-GTA GTG ATG CTT CTG CGA CAA-3, *Osteocalcin* (*Ocn*), tissue non-specific alkaline phosphatase (*Alpl*), *Klotho, FgfR1* and *FgfR3* (Qiagen; sequence not disclosed) and *Gapdh* (Primer Design, Southampton, UK; sequence not disclosed) were used.

2.7. Western blotting

Cultured cells were lysed in RIPA buffer (Invitrogen) containing "complete" protease inhibitor cocktail according to manufacturer's instructions (Roche). Immunoblotting was undertaken as previously described (MacRae et al., 2006b; MacRae et al., 2009). Recombinant mouse FGF-23 and Klotho were used as positive controls (R&D Systems). Nitrocellulose membranes were probed overnight at 4°C with anti-FGF-23 (R&D Systems), anti-Klotho (Abcam, Cambridge, UK), anti-FGFR1 (Cell Signaling Technology, Beverly, MA, USA) or anti-cleaved caspase 3 primary antibody (Cell Signaling Technology), washed in TBST and incubated with goat anti-rat (FGF-23) or goat anti-rabbit (Klotho, cleaved caspase-3 and FGFR1) IgG peroxidase secondary antibody (DAKO, Glostrup, Denmark) for 1h (1:3000 dilution in 5% BSA). The immune complexes were visualised using the enhanced chemi-luminescence (ECL) Western Blotting Detection System (GE Healthcare, Buckinghamshire, UK).

Membranes were then washed in Restore acidic antibody removal buffer (Pierce, Rockford, Il, USA) and re-probed for 1 h for β-actin expression (1:5000 dilution in 5% milk; anti β-actin-peroxidase clone AC15; Sigma).

2.8. Cell signalling immunoblotting

Following two days of culture following confluency, VSMCs were cultured for 24h in serum free medium, and then either lysed immediately or stimulated with FGF-23 (10ng/ml) for 10–60 min before lysis. Cells were lysed in PhosphoSafe extraction buffer (Merck Biosciences Ltd, Nottingham, UK) containing "Complete" protease inhibitor cocktail (Roche, East Sussex, UK) according to manufacturer's instructions. Immunoblotting was undertaken as previously described (MacRae et al., 2006b; MacRae et al., 2009). The membranes were probed for 1 hour at room temperature with primary antibodies raised in rabbit (all 1:1000 dilution in 5% milk) against phospho-Akt (ser 473), total Akt, phospho-P44/42 Map kinase (Thr202-/Tyr204) and total P44/42 Map kinase, (Cell Signaling Technology, Beverly, MA). The membranes were then incubated with anti-rabbit IgG-peroxidase (Cell Signalling Technology) for 1h (1:1000 dilution in 5% milk). The immune complexes were visualised as described above. Semi-quantitative assessment of band intensity was achieved using Quantity One image analysis software (Bio-Rad Labs, Inc., Hercules, CA).

2.9. Histology and immunohistochemistry

Aortae were dissected from 22 week old *Enpp1*$^{-/-}$ and WT mice. Tissues were fixed in 10% neutral buffered formalin for 10 min before being dehydrated and embedded in paraffin wax and sectioning at 4μm using standard procedures. Sections were de-waxed in xylene and stained with alizarin red (Sigma) to visualize calcium deposition. For immunohistochemistry, dewaxed sections were de-masked for 10min in 0.1% trypsin. Endogenous peroxidises and non-specific antibody binding were blocked before overnight incubation at 4°C with 5μg IgG/ml anti-FGF-23 antibody (R&D Systems) or 10μg IgG/ml anti-Klotho antibody (Abcam). The sections were then washed in PBS, incubated with goat anti-rat or goat anti-rabbit IgG peroxidise respectively (1:100 dilution, R&D Systems) for 1h, and incubated with DAB substrate reagent (0.06% DAB, 0.1% H_2O_2 in PBS) for 5min. The sections were finally dehydrated, counterstained with haematoxylin and eosin and mounted in DePeX.

Control sections were incubated with non-immune rat IgG (5μg IgG/ml) or non-immune rabbit IgG (10μg IgG/ml) in place of the primary FGF-23 or Klotho antibody, respectively.

2.10. Statistics

Data are presented as the means +/− S.E.M. Statistical analysis was determined by General Linear Model Analysis incorporating pairwise comparisons and the Student t-test using Minitab 15 (Minitab Inc, Coventry, UK). $P<0.05$ was considered to be significant.

3. Results

3.1. VSMC calcification in vitro is associated with increased FGF-23 expression

Alizarin red staining (calcium deposition) (Fig. 1A) and ALP activity (Fig. 1B) in aortic VSMCs were negligible at 0 d of culture. A significant and temporal increase in both matrix mineralization and ALP activity was noted following 7, 14 and 21 d of culture in calcifying medium containing βGP and AA. A significant increase in mRNA expression of *Alpl* (1.6 fold; P<0.001; Fig. 1C), *Runx2* (4.8 fold; P<0.001; Fig. 1D) and *PiT-1* (3.0 fold; P<0.001; Fig. 1E) was seen by 7 d and maintained for the duration of culture. Furthermore these osteogenic markers were significantly elevated in VSMCs cultured in calcifying medium, compared to corresponding mRNA derived from cells cultured in control medium (P<0.001). These results confirm the validity of this *in vitro* model to study *in vitro* calcification of aortic VSMCs over an extended culture period.

FGF-23, *Klotho* and *FgfR1* mRNA expression by VSMC was noted at all time-points during culture in calcifying medium containing βGP and AA (Fig 2). By 21 d, a significant increase in *FGF-23* expression (10.8 fold; P<0.05; Fig. 2A) was observed compared to 0 d. A significant increase in both *Klotho* (2.1 fold; P<0.05; Fig. 2B) and *FgfR1* (5.4 fold; P<0.001; Fig. 2C) mRNA expression was observed by 7 d compared to 0 d, which was maintained throughout the 21 d culture period. *FGF-23*, *Klotho* and *FgfR1* mRNA expression was significantly increased in cells cultured under calcifying conditions compared to VSMCs cultured under control conditions (P<0.001). Recombinant FGF-23 and Klotho were used to verify specificity of commercial antibodies (Fig. 2D). Bands corresponding to the size of both the FGF-23 and Klotho recombinant proteins were observed (30 and 125KD respectively), alongside the full length proteins present in VSMCs (37KD and 116KD respectively). The temporal FGF-23, FGFR1 and Klotho protein expression was generally comparable to corresponding gene expression (Fig. 2E). However, whilst FGFR1 mRNA expression was increased throughout the time course, no marked changes in FGFR1 protein were observed. This may be associated with post-transcriptional or post-translational regulation of FGFR1 expression.

Since medial vascular calcification is often due to increased phosphate levels, VSMCs were also cultured in growth medium for 14 days containing high P_i growth medium (3mM P_i). High P_i induced a significant increase in VSMC calcium deposition (determined by HCL leaching) at day 7 and day 14, compared to cells cultured in control medium (1mM P_i) ($P<0.001$; Fig. 3A). Notable differences in alizarin red staining were also observed by day 14 (Fig. 3A). A significant increase in mRNA expression of *Runx2* ($P<0.01$; Fig. 3B), *Alpl* ($P<0.01$; Fig. 3D) and *Fgf-23* ($P<0.05$; Fig. 3E) was seen by 7d and maintained for the duration of culture. *Pit-1* mRNA expression was significantly increased by 14d ($P<0.001$; Fig. 3C). Expression of Klotho and FGFR1 was observed at day 7 and 14 in both high P_i and control cultures (Fig. 3F). *FgfR3* expression was also noted in cultured VSMCs.

Together these studies show that calcification caused by elevated phosphate levels is associated with the up-regulation of FGF-23 expression in VSMCs, in the presence of FGFR1 and the cofactor Klotho.

3.2. Up-regulated FGF-23 and Klotho expression in calcified aortas from Enpp1$^{-/-}$ mice

The association of FGF-23 with vascular calcification was further strengthened by examination of the *Enpp1*$^{-/-}$ mouse. This mouse model presents with decreased levels of the mineralization inhibitor PP_i, with phenotypic features including significant alterations in bone mineralization in long bones and calvaria, and pathologic, severe perispinal soft tissue and moderate medial arterial calcification (Sali et al, 1999; Mackenzie et al., 2012a, 2012b). Calcification in the medial layer of the *Enpp1*$^{-/-}$ aorta was confirmed by alizarin red staining (Fig. 4A and 5A), with no staining observed in WT controls (Fig. 4B and 5B). Expression of FGF-23 and Klotho was also detected in the *Enpp1*$^{-/-}$ calcified aortic media (Fig. 4C and Fig. 5C respectively). No positive staining for FGF-23 was seen in WT mice (Fig. 4D) respectively or control sections incubated with IgG only (Fig. 4E&F). However, basal levels of Klotho expression were observed in WT mice (Fig. 5D). Expression of *FgfR1* was also confirmed in *Enpp1*$^{-/-}$ and WT aortae. Interestingly, reduced *FgfR1* expression was noted in the *Enpp1*$^{-/-}$ tissue (Fig. 5G). These immunolocalisation studies verify our *in vitro* data, and confirm that up-regulation of FGF-23 and Klotho is associated with the vascular calcification process.

3.3. FGF-23 directly modulates VSMC calcification

Having established the increased expression of FGF-23 during the VSMC calcification process, we sought to establish whether FGF-23 promotes or inhibits vascular calcification by direct treatment of VSMCs with recombinant FGF-23. Since medial vascular calcification is often due to increased phosphate levels, further studies were undertaken in VSMCs cultured for 9 days in growth medium containing 3mM P_i in the presence of FGF-23 (10 and 50ng/ml). No effect of treatment was noted in low Pi medium (1mM P_i). A significant reduction in calcium deposition was observed following FGF-23 treatment at both 10ng/ml (28.1% decrease; $P<0.01$; Fig. 6A) and 50ng/ml (28.8% decrease; $P<0.01$; Fig. 6A). Furthermore, FGF-23 treatment induced a significant reduction in the mRNA expression of osteogenic markers *Ocn* ($P<0.05$; Fig. 6B) and *Pit-1* ($P<0.05$; Fig. 6C). No change in *Runx2* or *Alpl* mRNA expression was observed (Fig. 6C). No effect of FGF-23 treatment on cell viability (Fig. 6D) or cleaved caspase-3 expression (as an indication of apoptosis) was noted (Fig. 6E). To further investigate the functional role of FGF-23 signalling in vascular calcification, VSMCs were treated with the FGFR1 and FGFR3 inhibitor, PD173074. A significant increase in calcification was demonstrated following PD173074 treatment at both 10nM (37.6% increase; $P<0.01$) and 50nM (87.8% increase; $P<0.001$; Fig. 7A). No effect of PD173074 treatment on cell viability was observed (Fig. 7B) Taken together these data suggest that the stimulation of FGF-23 signaling is a protective mechanism against VSMC calcification.

3.4. FGF-23 prevents VSMC calcification through activation of the Erk1/2 pathway

Previous studies have shown that FGF-23 increases Akt and Erk1/2 phosphorylation in both renal proximal tubule epithelial cells (Medici et al., 2008) and human aortic smooth muscle cells (Lim et al., 2012). Signal transduction studies were therefore completed to disclose the FGF-23 initiated signaling mechanism by which this ligand prevents VSMC calcification. FGF-23 significantly induced phosphorylation of Erk1/2, after 10 min ($P<0.01$) and 30 min ($P<0.05$) but not after 60 min exposure (Fig. 8A & B). In contrast, Akt phosphorylation was not induced following FGF-23 treatment at any of the time points studied (Fig 8A & C). These data potentially suggest that FGF-23 may prevent VSMC calcification via ERk1/2 signaling and therefore to test this directly we next treated VSMC with FGF-23 alone or in combination with PD98059 (10μM), an Erk1/2 inhibitor (MacRae et al., 2007).

As previously shown (Fig. 6A) FGF-23 significantly prevented VSMC calcification in comparison to control cultures (Fig 8D) and this reduction was reduced when PD98059 was also present, resulting in a complete ablation of the protective effect afforded by FGF-23 treatment (Fig. 8D). PD98059 had no significant effect on VSMC calcification when added alone (Fig. 8D). Together these data strongly support the notion that activation of the Erk1/2 pathway is a key role in mediating the protective effect of FGF-23 against VSMC calcification.

4. Discussion

Currently, there is conflicting evidence as to whether FGF-23 imparts a protective or a harmful role on the vasculature during stress, with recent studies suggesting that FGF-23 directly inhibits vascular calcification (Lim et al., 2012; Razzaque and Lanske, 2007; Shalhoub et al., 2012). Our studies in mice support and extend these findings, and indicate that FGF-23 is directly involved in modulating the pathogenesis of vascular calcification by exerting a protective effect on arterial wall integrity through activation of the Erk1/2 pathway.

In the present study, quantitative alizarin red staining of calcium deposition confirmed the formation of calcified matrix in murine VSMC cultures, induced by treatment with ascorbic acid and βGP. This is in agreement with previous reports by our laboratory (Zhu et al., 2011). In addition, VSMCs cultured under calcifying conditions showed increased ALP activity and expression of Runx2 and P_iT-1, which are recognised regulators of vascular calcification (Mackenzie et al., 2011; Speer et al., 2009; Zhu et al., 2011).

In order to investigate the association between FGF-23 and vascular calcification, the expression patterns of FGF-23 and Klotho, the co-factor required for FGF-23-dependent FGFR1 activation, were determined during VSMC calcification *in vitro*. Increased expression levels of *FGF-23* and *Klotho* mRNA were noted after 7 days of culture in calcifying VSMCs. This increased expression was maintained throughout the culture period. Comparable changes in protein expression were also observed. FGFR1 expression was also confirmed in these cells, establishing the potential for FGF-23 to directly function in VSMCs through binding to FGFR1 in the presence of Klotho.

This study is the first to demonstrate *FgfR3* expression in murine VSMCs. It has recently been shown that not only FGFR1, but also FGFR3 is expressed in human vascular tissue (Donate-Correa et al., 2011; Lim et al., 2012). Furthermore, it has demonstrated that expression of FGFR1 and FGFR3 in human arteries from healthy individuals and CKD patients is critical for vascular calcification, and that the physical association of Klotho, FGFR1 and FGFR3 is an essential mechanism to prevent critical vascular calcification (Lim et al., 2012).

To our knowledge, this is the first report indicating that FGF-23 and Klotho are up-regulated during murine VSMC calcification *in vitro*. These data were confirmed and extended by studying an *in vivo* mouse model of vascular calcification. Mice lacking ecto-nucleotide pyrophosphatase/phosphodiesterases-1 (NPP1, a.k.a PC-1), a major generator of extracellular PP_i, spontaneously develop articular cartilage, perispinal, and medial aortic calcification at a young age (Mackenzie et al., 2012a, 2012b; Sali et al., 1999). These NPP1 knockout mice ($Enpp1^{-/-}$) share phenotypic features with a human disease, idiopathic infantile arterial calcification, and show elevated levels of circulating FGF-23 (Mackenzie et al., 2012a, 2012b; Rutsch et al., 2001, 2003). In the present study, our immunohistochemical approach demonstrated increased expression of FGF-23 and Klotho in the calcified media of $Enpp1^{-/-}$ aortic tissue. This data is supported by the expression of FGF-23 within calcified areas of atherosclerotic lesions (Voigt et al., 2010). However, the reduced levels of PP_i in this mouse model may generate different effects on the vascular phenotype to that seen in alternative animal models of vascular calcification, such as kidney failure models. Additionally, the demonstration of the osteocytic hormone FGF-23 in $Enpp1^{-/-}$tissue confirms and extends recent data demonstrating the up-regulation of molecules such as sclerostin associated with the osteocyte phenotype in the $Enpp1^{-/-}$mouse model of aortic medial calcification (Zhu et al., 2011). Our demonstration of Klotho expression in the rodent vasculature extends recent data describing Klotho expression in human arteries and aortic smooth muscle cells (Donate-Correa et al., 2011; Lim et al., 2012). It has recently been demonstrated that CKD is a state of vascular Klotho deficiency promoted by chronic circulating stress factors, including pro-inflammatory, uremic and disordered metabolic conditions (Lim et al., 2012). Vascular produced Klotho has been shown to be an endogenous inhibitor of calcification (Lim et al., 2012), and may therefore be up-regulated in an attempt to impart, together with FGF-23, a protective function in $Enpp1^{-/-}$tissue. Indeed emerging evidence now suggests that Klotho exerts direct cardiovasculo-protective effects (Hu et al., 2011; Liu et al., 2011; Rakugi et al., 2007; Saito et al., 2000; Yamamoto et al., 2005), revealing a new mechanism by which Klotho may exert anti-aging effects in the arterial system.

In order to establish whether FGF-23 is directly involved in modulating the pathogenesis of vascular calcification, functional studies on VSMCs were undertaken *in vitro*. FGF-23 treatment inhibited calcium deposition, with a concomitant reduction in the expression of osteogenic markers osteocalcin and Pit-1. Interestingly, *Alpl* expression remained unaltered, suggesting the involvement of an ALP-independent mechanism, which requires further investigation. Treatment of cells with recombinant FGF-23 Administration of the FGFR inhibitor PD173074 to cultured VSMCs promoted phosphate-stimulated calcification. PD173074 has been previously shown to completely block the stimulation of the *Fgf-23* promoter in osteoblastic cells (Liu et al., 2009). Our data therefore suggests that the augmented FGF-23 produced during VSMC calcification exerts a protective effect through binding to FGF receptors. Further studies demonstrated a direct inhibitory action of FGF-23 treatment on VSMC calcification *in vitro*. These findings support and extend recent studies that have demonstrated that FGF-23 is able to significantly inhibit extracellular calcium deposition in human aortic VSMCs after pre-treatment with calcitrol (Lim et al., 2012). Notably, Lim and colleagues also found that these beneficial effects were shown to be reversed after suppressing Klotho protein synthesis with Klotho siRNA. A vasculo-protective role for FGF-23 is supported by the observation that *Fgf-23* null mice show extensive vascular and soft tissue calcification, together with severe hyperphosphatemia (Shimada et al., 2004; Sitara et al., 2004). There is also controversial evidence that FGF-23 may directly inhibit skeletal calcification, independent of phosphate homeostasis (Sitara et al., 2008). Furthermore, inactivating mutations of FGF-23 in diseases such as familial tumoral calcinosis manifest with severe ectopic calcification (Razzaque and Lanske, 2007). However, contrary to these reports is the observation that increased FGF-23 plasma levels in

patients with CKD, have been linked to decreased kidney and vascular function, and increased risk of cardiovascular mortality (Isakova et al., 2011; Nashrallah et al., 2010; Parker et al., 2010; Shrivaths et al., 2011; Yilmaz et al., 2010). It has been proposed that reduced Klotho tissue levels cause vascular resistance to FGF-23 in CKD, thus masking FGF-23's protective effects on the vasculature (Lim et al., 2012).

In order to elucidate the potential mechanism through which FGF-23 may be exerting it's protective effect on VSMCs, modulation of the PI3-kinase/Akt and MAPK/Erk1/2 signalling pathways were examined. These pathways are involved in a wide range of cellular functions, including transcription, proliferation, migration, survival, differentiation and calcification (Kok et al., 2009; Roy et al., 2001; Salasznyk et al., 2004). Our data revealed that FGF-23 only induced the phosphorylation of Erk1/2 in murine VSMCs, corroborating the recent report describing FGF-23 activation of this pathway in human aortic smooth muscle cells (Lim et al., 2012). Interestingly, in contrast to the studies undertaken by Lim and colleagues (Lim et al., 2012), no induction of Akt phosphorylation was noted. This may be due to the differences in cell culture conditions and/or divergent human and mouse VSMC responses to FGF-23. In this present study we demonstrate that the inhibition of the Erk1/2 pathway results in the complete loss of the protective effect against VSMC calcification afforded by FGF-23 treatment. The Erk1/2 pathway has been shown to regulate calcification in various osteoblast (bone forming) and osteoblast precursor cell types (Franceschi et al., 2007; Khatiwala et al., 2007; Salasznyk et al., 2004). Consistent with these actions, several studies have shown that Erk1/2 activation regulates calcification and osteoblastic differentiation in vascular smooth muscle cell cultures (Ding et al., 2006; Roy et al., 2001; Simmons et al., 2004). Our data therefore confirms and extends these previous reports demonstrating the importance of the Erk1/2 pathway in regulating vascular calcification.

5. Conclusions

In the present study, we have undertaken *in vitro* and *ex vivo* murine VSMC calcification studies to provide fundamental insights into the expression profiles of FGF-23 during vascular calcification. Our studies suggest that the Erk1/2 signalling pathway is essential for FGF-23 to prevent murine VSMC calcification *in vitro*. FGF-23 therefore appears to play a critical role in vascular calcification, and may represent a novel potential therapeutic strategy for clinical intervention.

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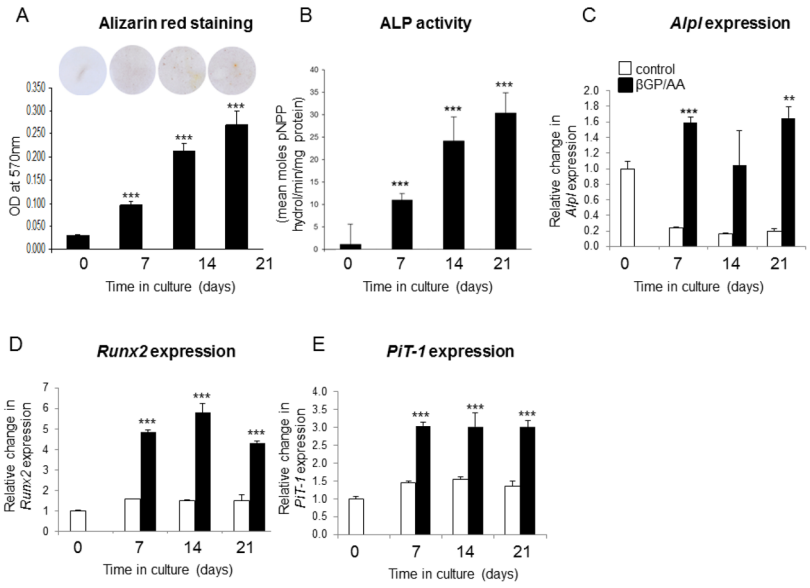

Figure 1. *In vitro* calcification of murine aortic VSMCs cultured for 21 d under calcifying conditions

Quantification of (A) Alizarin red staining and (B) Alkaline phosphatase activity (mean moles pNPP hydrol/min/mg protein) (n=3). Fold change in (C) *Alpl*, (D) *Runx2* and (E) *P_iT-1* RNA expression in VSMCs cultured with 2.5 mM βGP and 50 μg/ml ascorbic acid or control medium (n=4). VSMCs were treated with control (white bar) or βGP/AA containing (filled bar) medium. Results are presented as mean+/− S.E.M. **P<0.01; ***P<0.001 compared with day 0.

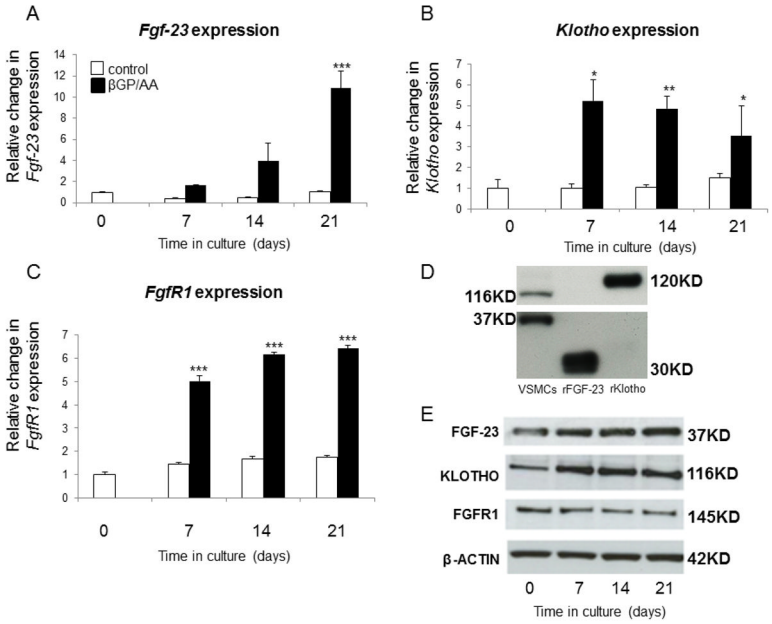

Figure 2. Up-regulation of FGF-23 expression during *in vitro* calcification of murine aortic VSMCs cultured for 21d with βGP and ascorbic acid

Fold change in the mRNA expression of (A) *FGF-23*, (B) *Klotho* and (C) *FgfR1*. (D) Recombinant mouse FGF-23 (rFGF-23) and Klotho (rKlotho) were used to verify specificity of commercial antibodies in VSMCs (E) FGF-23, Klotho and FGFR1 protein expression in corresponding VSMCs cultured with 2.5 mM βGP and 50 μg/ml ascorbic acid. VSMCs were treated with control (white bar) or βGP/AA containing (filled bar) medium. Results are presented as mean+/− S.E.M., n=4 *P<0.05; **P<0.01; ***P<0.001 compared with day 0.

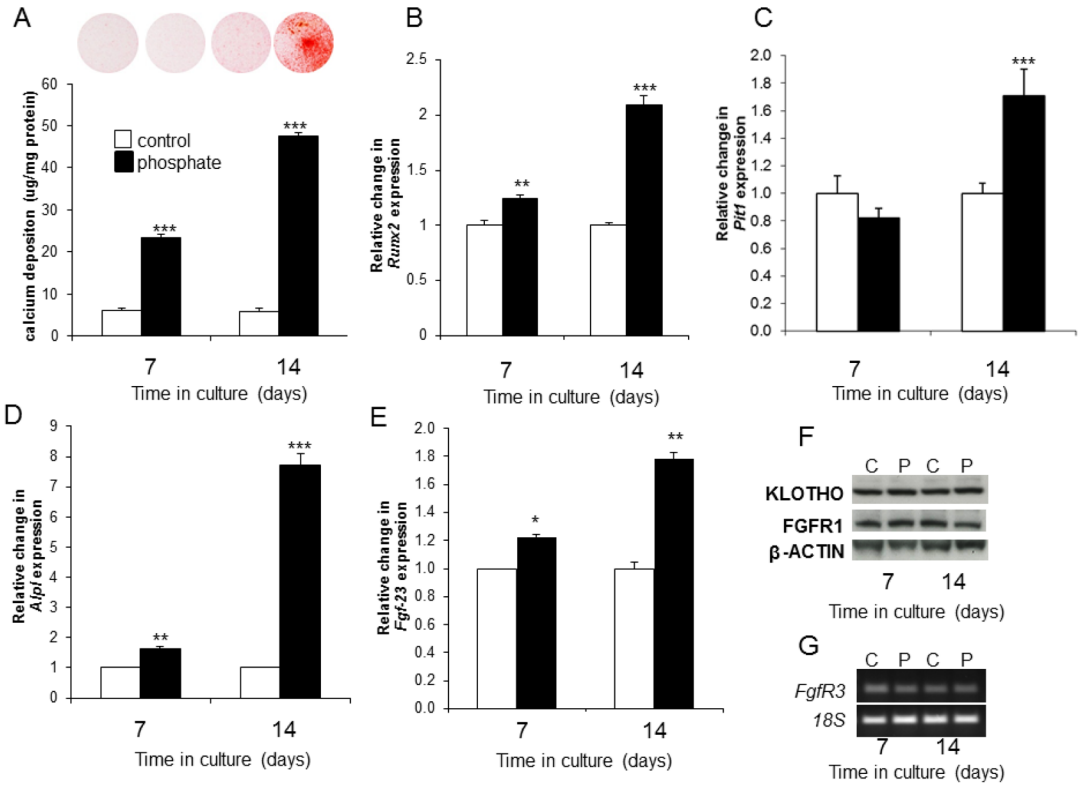

Figure 3. Up-regulation of FGF-23 expression during *in vitro* calcification of murine aortic VSMCs cultured for 14d with 3mM P_i

(A) Calcium content was determined by quantification HCL leaching (μg/mg protein) (n=3), and visualised with alizarin red staining. Fold change in the mRNA expression of (B) *Runx2*, (C) *Pit-1* (D) *Alpl* and (E) *Fgf-23*. VSMCs were treated with control (white bar) or βGP/AA containing (filled bar) medium. (F) Klotho and FGFR1 protein expression and (G) *FgfR3* expression in corresponding VSMCs cultured with control [C] or 3mM P_i [P] medium (n=4). Results are presented as mean+/− S.E.M. *P<0.05; **P<0.01; ***P<0.001 compared with day 0.

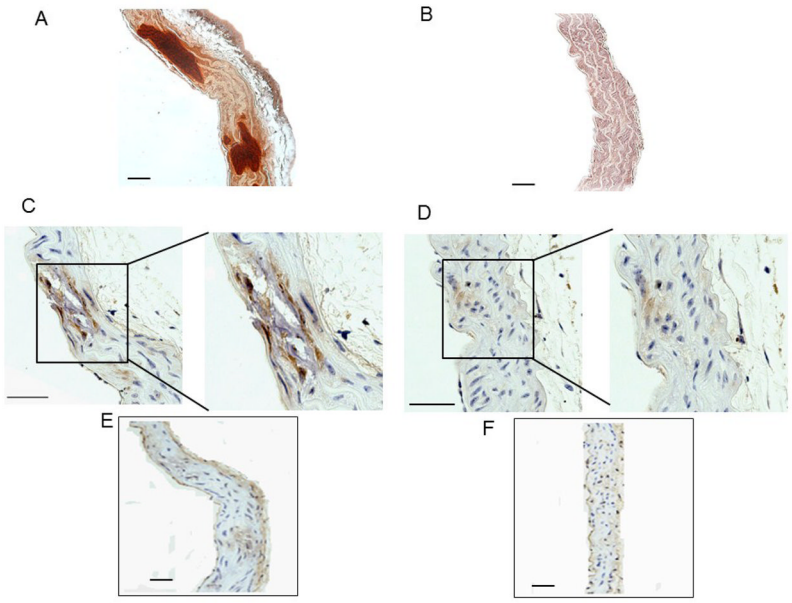

Figure 4. Emergence of FGF-23 in the calcified aorta from the *Enpp1*$^{-/-}$ mouse *in vivo*

Medial aortic calcification was detected by alizarin red staining in (A) *Enpp1*$^{-/-}$ tissue compared to (B) *Enpp1*$^{+/+}$ control. (C) Increased protein expression of FGF-23 was observed in the calcified media of *Enpp1*$^{-/-}$ aortic tissue (arrows). (D) No expression of FGF-23 was detected in the non-calcified media of the *Enpp1*$^{+/+}$ control. Representative image of (E) *Enpp1*$^{-/-}$ compared to (F) *Enpp1*$^{+/+}$ negative control tissue. Scare bars = 50μm.

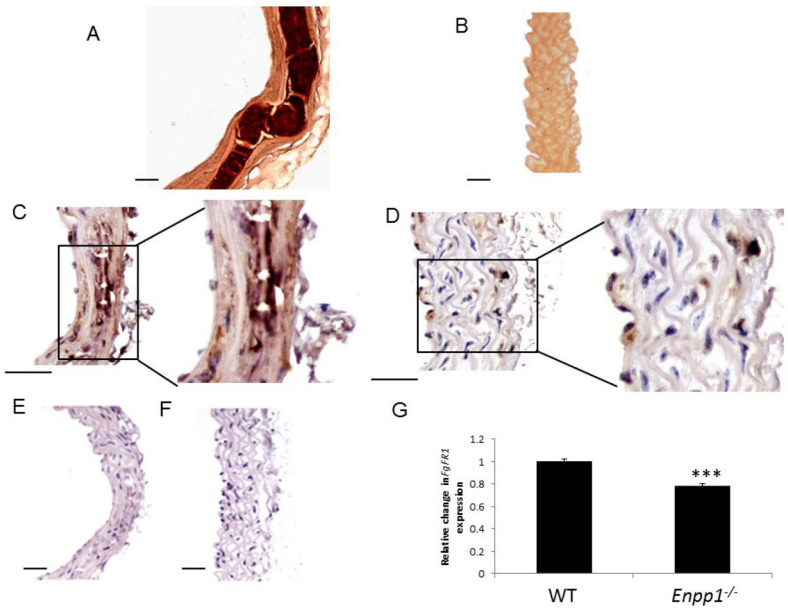

Figure 5. Emergence of Klotho in the calcified aorta from the *Enpp1*$^{-/-}$ mouse *in vivo*

Medial aortic calcification was detected by alizarin red staining in (A) *Enpp1*$^{-/-}$ tissue compared to (B) *Enpp1*$^{+/+}$ control. (C) Increased protein expression of Klotho was observed in the calcified media of *Enpp1*$^{-/-}$ aortic tissue. (D) Basal expression levels of Klotho was detected in the non-calcified media of the *Enpp1*$^{+/+}$ control. Representative image of (E) *Enpp1*$^{-/-}$ compared to (F) *Enpp1*$^{+/+}$ negative control tissue. (G) Fold change in the mRNA expression of *FgfR1* in *Enpp1*$^{-/-}$ VSMCs compared to *Enpp1*$^{+/+}$ control cells. Scare bars = 50μm.

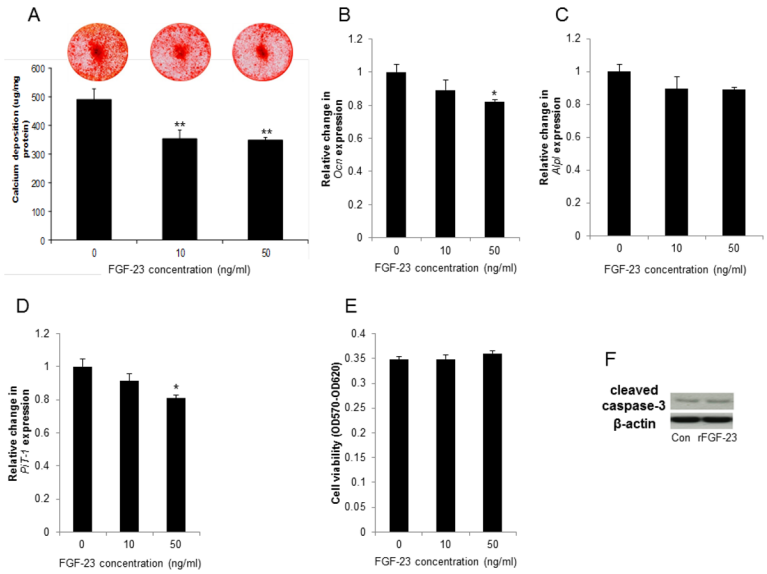

Figure 6. Effect of FGF-23 treatment on the *in vitro* calcification of murine aortic VSMCs

VSMCs were incubated with FGF-23 (10–50ng/ml) in the presence of 3mM P_i for 9 days. (A) Calcium content was determined by quantification HCL leaching (μg/mg protein) (n=5), and visualised with alizarin red staining. Fold change in the mRNA expression of osteogenic markers (B) *Ocn* (C) *Alpl* and (D) *Pit-1* (n=4). (E) Cell viability was assessed using the Alamar blue assay (OD570–OD620) (n=6). (F) Expression of cleaved caspase-3 protein as an indication of apoptosis. Results are presented as mean+/− S.E.M. *P<0.05; **P<0.01 compared with control.

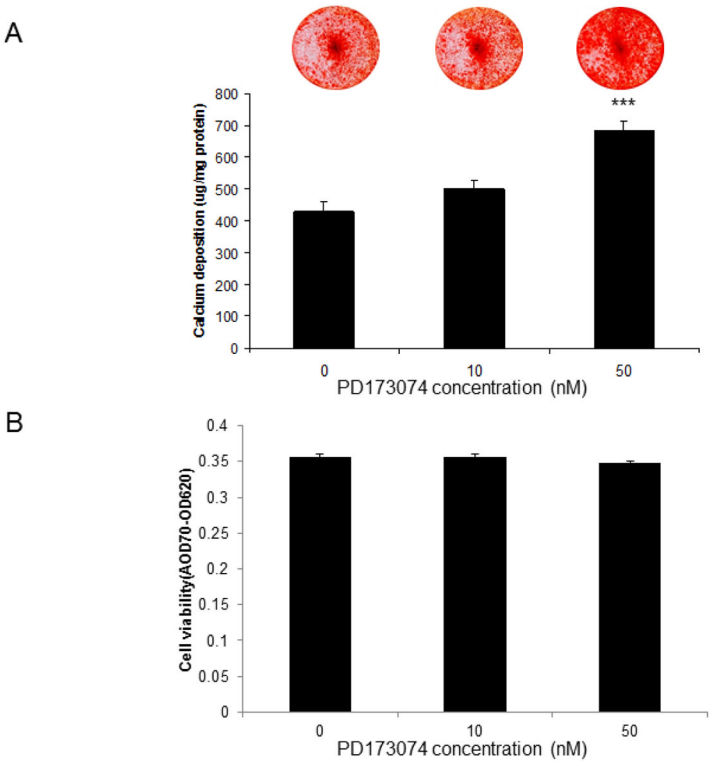

Figure 7. Effect of FGFR1 inhibition on the *in vitro* calcification of murine aortic VSMCs

VSMCs were incubated with the FGFR1 inhibitor PD173074 (10–50nM) in the presence of 3mM P_i for 9 days. (A) Calcium content was determined by quantification HCL leaching (μg/mg protein) (n=5), and visualised with alizarin red staining. (B) Cell viability was assessed using the Alamar blue assay (OD570–OD620) (n=6). Results are presented as mean +/− S.E.M. ***P<0.001 compared with control.

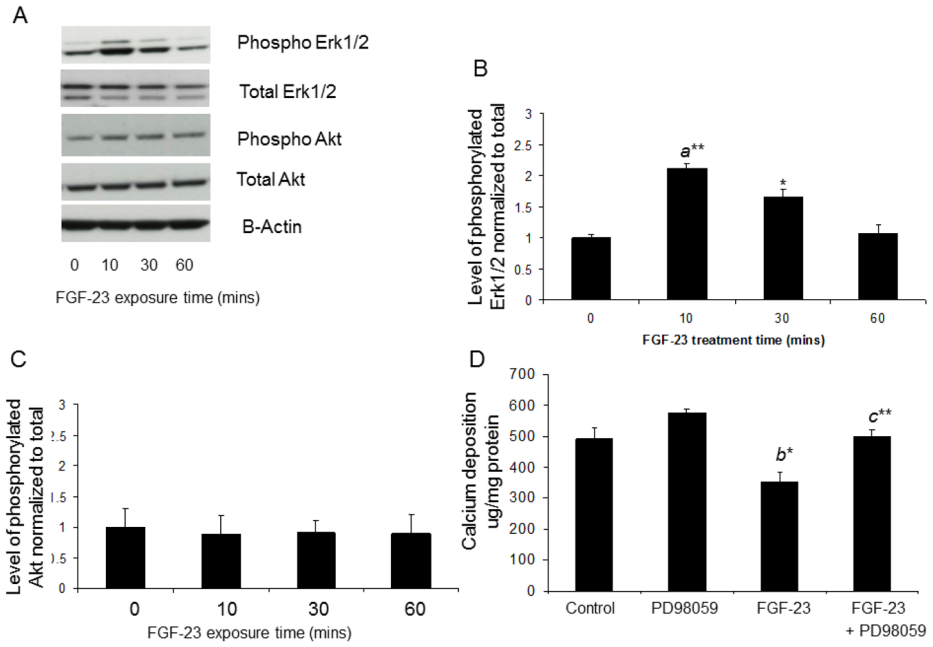

Figure 8. FGF-23 induces Erk 1/2 activation in VSMCs

Effect of FGF-23 (10ng/ml) on the phosphorylation of Akt compared with total Akt, and Erk1/2 compared with total Erk1/2 shown by (A) western blot analysis and (B,C) densitometry quantification (n=3). (D) Effect of PD98059 (10μM) on calcium content (μg/mg protein) of VSMCs in the presence/absence of FGF-23 (10ng/ml) (n=5). Results are presented as mean+/− S.E.M. ** P<0.01; * P<0.05 compared with *a* 0 exposure time (mins); *b* control and *c* FGF-23.